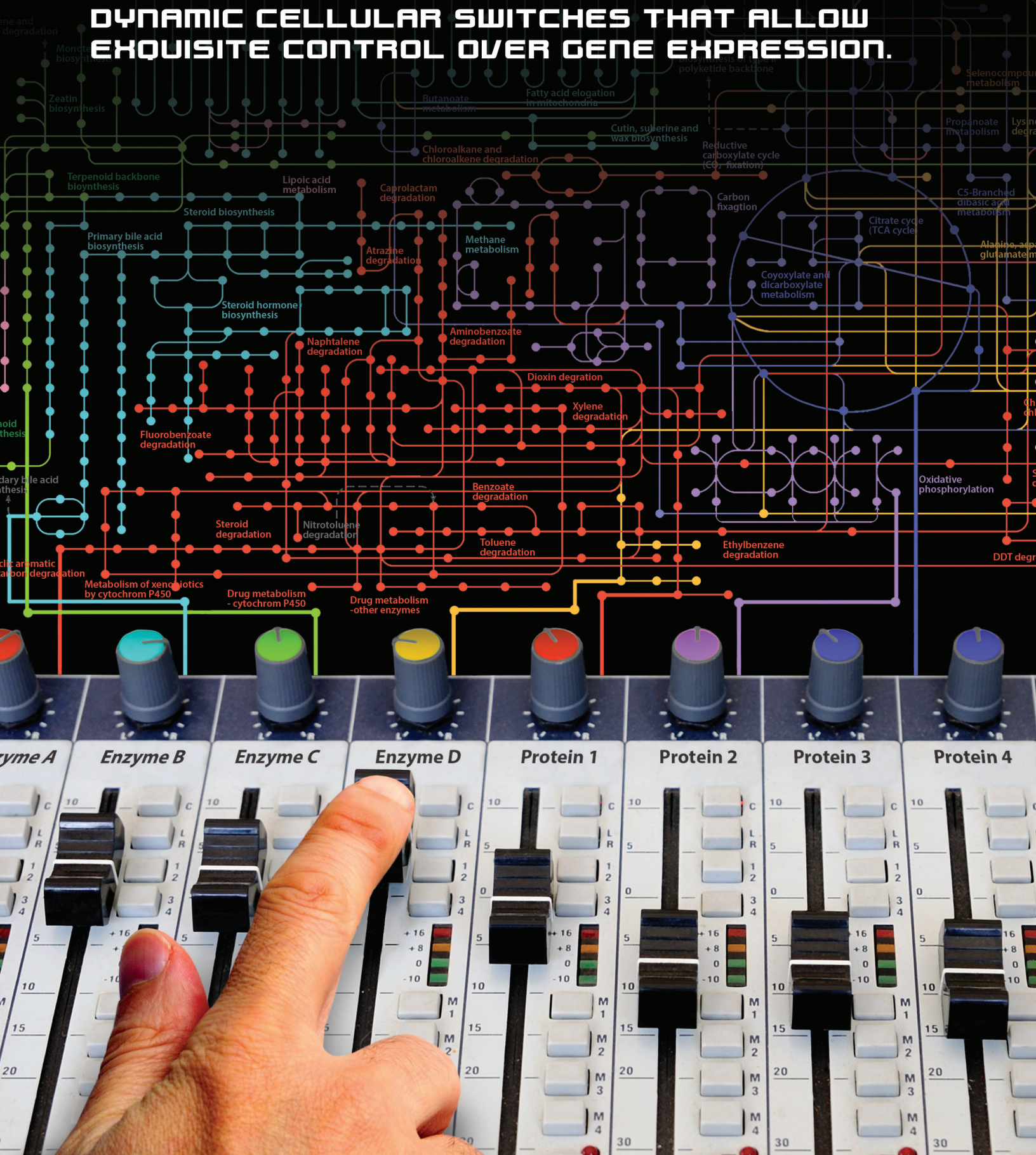
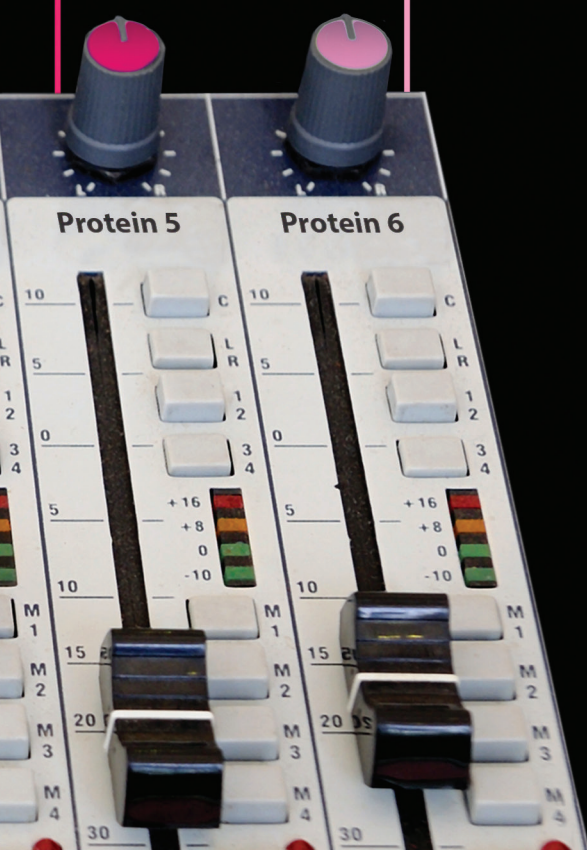
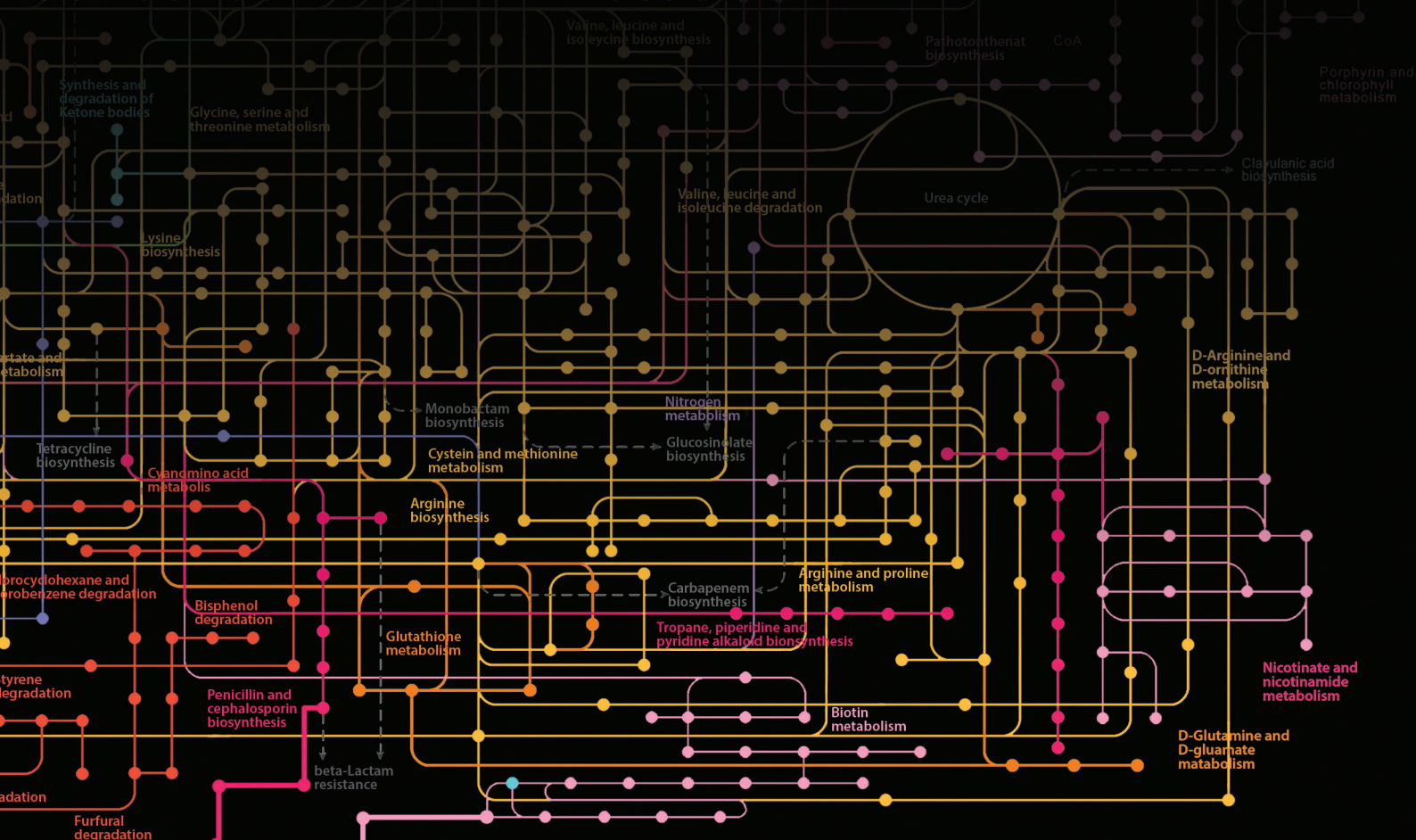


# MASTERING

**DYNAMIC CELLULAR SWITCHES THAT ALLOW  
EXQUISITE CONTROL OVER GENE EXPRESSION.**



# THE MIX



THE STUFF OF LIFE is widely agreed to be deoxyribonucleic acid, or DNA. The stalwart double helix has become iconic as the molecule that encodes and ensures a living thing's genetic stock. But add two atoms—an oxygen and a hydrogen—and instead of DNA you get its wily sibling, ribonucleic acid, or RNA. Arguably the more charismatic of the two nucleic acids, RNA is less familiar than DNA outside of scientific circles.

Established dogma simplifies RNA to the intermediary between DNA and protein—the gofer that shuttles protein production instructions from a cell's nucleus to its outlying and scattered protein production factories. But RNA is far more involved than that. Yes, the messenger that carries instructions from the nucleus is made of RNA. But so is the message itself, the protein production machine receiving the message, and also the handler molecules that wrangle protein building blocks from the cellular ephemera and deliver them into that machine. Many viruses use only RNA to encode their genomes, and RNA regulates gene expression in higher organisms at just about every step along the way from mitosis to apoptosis—that is, from a cell's creation to its destruction.

Because RNA is such a central player to the processes of life, it is the subject of much study. But it's very complex—far more complex than DNA. Whereas DNA usually takes a single shape, the double helix, RNA takes all sorts of different shapes, and its functionality comes from the shape it takes. Predicting an RNA

# A tale of two nucleic acids



In the utility drawer of cellular machinery, DNA is like a spoon: stable, predictable, the absolute right tool for a very specific job. RNA is like a Swiss army knife: versatile, delicate, taking myriad forms to tackle numerous jobs. Whereas DNA is usually a double-stranded helix, with each complementary strand twisting around the other to provide strength, structure, and reliability, RNA is typically single stranded. But, biochemically, it still “wants” to pair up. So with no sister strand to bind to, it kinks and twirls back on itself forming elaborate loops, bulges, hairpins, and helices.

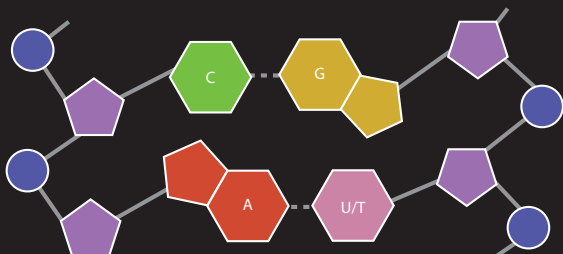
DNA and RNA both participate in base pairing, a process in which the nitrogenous bases on one strand share hydrogen atoms with the bases of another strand, forming hydrogen bonds that bridge the gap and hold the two strands together. Generally speaking, the four bases of RNA follow the rule that cytosine (C) pairs with guanine (G) and adenine (A) pairs with uracil (U) (in DNA base pairing, adenine pairs with thymine (T)). But there's some flexibility to that rule.

Because G and A are similar to each other in size and shape (belonging to a class of double-ringed molecules called purines), and C and U are similar to each other (belonging to a class of single-ringed molecules called pyrimidines), they can occasionally swap partners. So G can sometimes pair with U and C can sometimes pair with A. This flexible base pairing is a major source of RNA's versatility.

For an RNA molecule, structure and function are inextricably linked, and often one can be predicted from the other. How an RNA molecule base pairs with itself or with other RNA molecules is referred to as its secondary structure (the primary structure being the sequence of C's, G's, A's, and U's that make up the RNA molecule). Tertiary structure, then, has to do with the three-dimensional shape of the folded-up RNA molecule. Secondary and tertiary structure affect how an RNA molecule can interact with other molecules—where it can fit and what it can bind to—and so determine what it can do.

Depending on a number of factors, the same primary structure can result in different secondary and tertiary structures, which impart different functions. Both regulatory mechanisms being studied by the Los Alamos group, riboswitches and riboregulators, capitalize on this interchange to control gene expression.

The two strands of a DNA molecule (pink) pair with and bind to each other. The single strand of an RNA molecule (blue) pairs with and binds to itself, forming elaborate structures that impart the molecule's many functions.



Both DNA and RNA undergo base pairing, wherein a purine (A or G) hydrogen bonds with an opposing pyrimidine (C or U/T). Usually C pairs with G and A pairs with U/T, but partner swapping can occur and contributes to RNA's versatility.

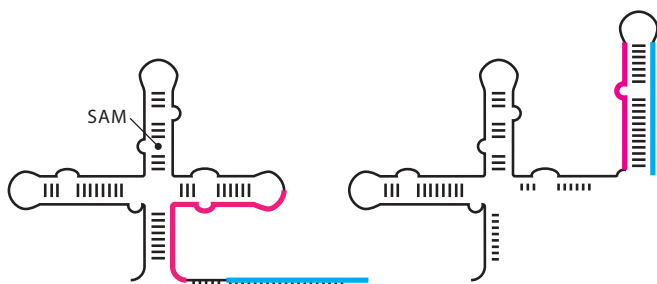
molecule's 3D shape, the way it folds and loops and coils, is notoriously difficult. But by using its genetic sequence (primary structure) as a starting point, scientists are getting better at mathematically predicting these complex secondary and tertiary structures through computer simulation.

**IN THE CELLULAR UTILITY DRAWER, DNA IS LIKE A SPOON, AND RNA IS LIKE A SWISS ARMY KNIFE.**

Taking a combinational approach—mathematical simulation and biochemical experimentation—Los Alamos researchers are capitalizing on RNA's versatility to adjust bacterial metabolism for a variety of far-reaching goals, such as the design of new drugs, environmental clean up, and even energy production. Through the discovery of natural tricks and invention of new tactics, they are harnessing the power of RNA to help dial in and fine tune the right metabolic mix.

## Natural tricks

Theoretical biologist Karissa Sanbonmatsu models RNA molecules and their mechanisms to better understand how they work. Having built a previous successful career in plasma physics, Sanbonmatsu was drawn to biology in general, and RNA modeling in particular, by the enigmatic ribosome. Made mostly of intricately folded RNA, ribosomes are the molecular machines that assemble proteins based on RNA instructions called transcripts. Much of the work in Sanbonmatsu's lab is focused on ribosomes, and the group is a world leader in ribosome modeling. But lately, the group has also been



The mechanism of a riboswitch from the hot-springs-dwelling species of bacteria *Thermoanaerobacter tengcongensis*. Through variable base pairing, the same RNA molecule can change shape in order to regulate gene transcription. (Left) When the ligand molecule SAM (black dot) is bound to the riboswitch, the RNA takes a form (pink not paired with blue) that prevents gene transcription. (Right) When the ligand molecule is not present, the riboswitch takes a different conformation (pink paired with blue) that allows gene transcription. The two structures have the exact same genetic sequence, but the binding of the ligand favors one conformation over the other.

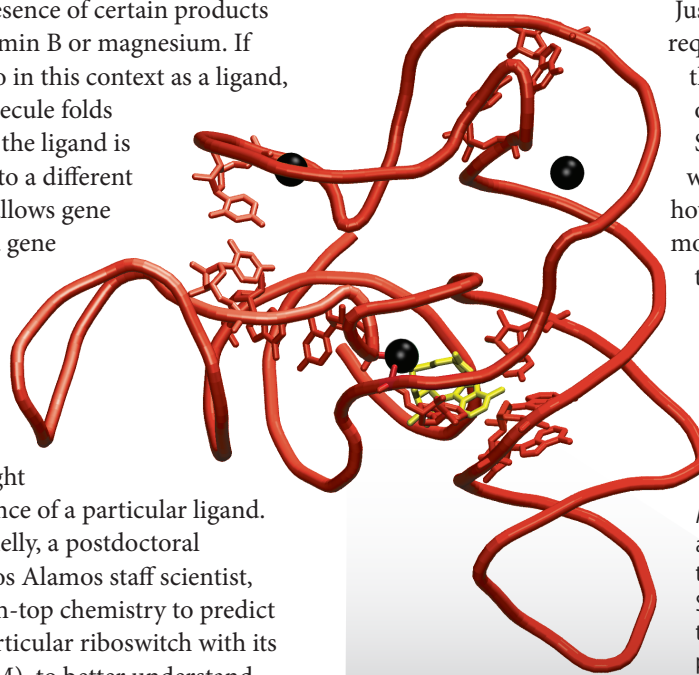
working on a different class of RNA machines, subcellular stoplights called riboswitches.

Naturally occurring in bacteria, riboswitches are small RNAs that are controlled by the presence of certain products of normal metabolism, such as vitamin B or magnesium. If the metabolism product, referred to in this context as a ligand, is bound to the RNA, the RNA molecule folds into one shape, or conformation; if the ligand is not present, the same RNA folds into a different conformation. One conformation allows gene transcription—the first step toward gene expression, wherein a transcript, or copy, of the DNA gene is constructed from RNA—and the other conformation blocks gene transcription. So the riboswitch is essentially a regulator of gene expression, giving a green or red light depending on the presence or absence of a particular ligand.

Sanbonmatsu and Scott Hennelly, a postdoctoral researcher at the time and now a Los Alamos staff scientist, used computer modeling and bench-top chemistry to predict and validate the interaction of a particular riboswitch with its ligand, S-adenosylmethionine (SAM), to better understand how riboswitches work. SAM regulates sulfur metabolism, participates in gene regulation, and directs its own production via riboswitch. The SAM riboswitch operates by competitive mutual exclusion: through flexible base pairing, a portion of its sequence (the C's, G's, A's and U's) can pair with either of two nearby regions of the same RNA molecule. Which one it pairs with determines whether the switch is in the on or off position. When concentrations of the ligand SAM are high, SAM binds to the riboswitch, which prevents the transcription of the gene encoding SAM synthetase (the enzyme responsible for activating SAM), so the switch is off and no more SAM gets made. When the concentration of SAM is low, the SAM-less riboswitch takes a conformation that permits the transcription of the SAM synthetase gene, thus flipping the switch on and allowing for the production of more SAM.

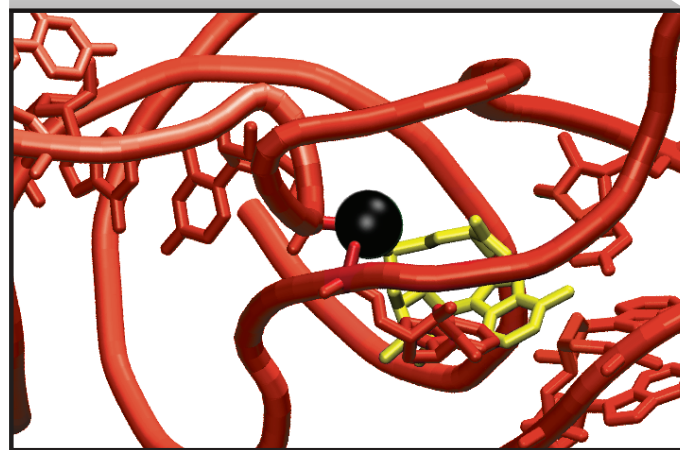
And if that's not convoluted enough, it gets better—because RNA structures often require the presence of certain ions for correct formation. Sanbonmatsu and Hennelly found that in addition to SAM binding, the transcription-blocking conformation of the SAM riboswitch depends on the presence of divalent magnesium ( $Mg^{2+}$ ). The “transcription-off” position requires SAM and three molecules of  $Mg^{2+}$  bound to three specific locations on the RNA. But puzzlingly, very high concentrations of  $Mg^{2+}$  can induce the riboswitch to the “transcription-on” position despite SAM being bound to the RNA. Additional experiments show that modifying any of these three sites so that  $Mg^{2+}$  can't bind also made SAM less able to bind, thereby destabilizing the “transcription-off” conformation. Sanbonmatsu and Hennelly conclude that there must be a cooperation between SAM and  $Mg^{2+}$  where SAM magnifies its ability to control the structure of the riboswitch by recruiting  $Mg^{2+}$ .

“We think SAM acts like a crowbar to pry open certain sites so that  $Mg^{2+}$  can bind, and at physiological concentrations, this shuts down transcription,” Hennelly explains.



Just knowing that  $Mg^{2+}$  is required, though, isn't the end of the story. In fact it's kind of a new beginning. Next, Sanbonmatsu and her team will plunge into figuring out how  $Mg^{2+}$  and SAM cooperate molecularly and exactly what they do inside the SAM riboswitch. How does the presence or absence of

A three-dimensional view of the SAM and  $Mg^{2+}$  binding sites on a portion of the SAM riboswitch. The ligand molecule SAM (yellow) is in the center, and the three magnesium ions (black) are on the periphery.



Mg<sup>2+</sup> determine how well SAM can bind? What about free-floating Mg<sup>2+</sup> ions—do they have any influence? And just how is it that excessive quantities of Mg<sup>2+</sup> can override SAM? In collaboration with researchers at Rice University, the team is working on faster new models and fancier new algorithms that will start to answer some of these questions.

“While we know for the most part what RNA does,” says Sanbonmatsu, “we have a long way to go to understand how it does it. Understanding the ‘how’ is going to be the key to some blockbuster new discoveries.”

## New tactics

Bacteria evolved the riboswitch mechanism of RNA-based gene regulation all on their own. But Los Alamos biochemist Cliff Unkefer, along with Sanbonmatsu, Hennelly, and a team of others, is taking the idea that RNA makes a good control switch to a whole new level.

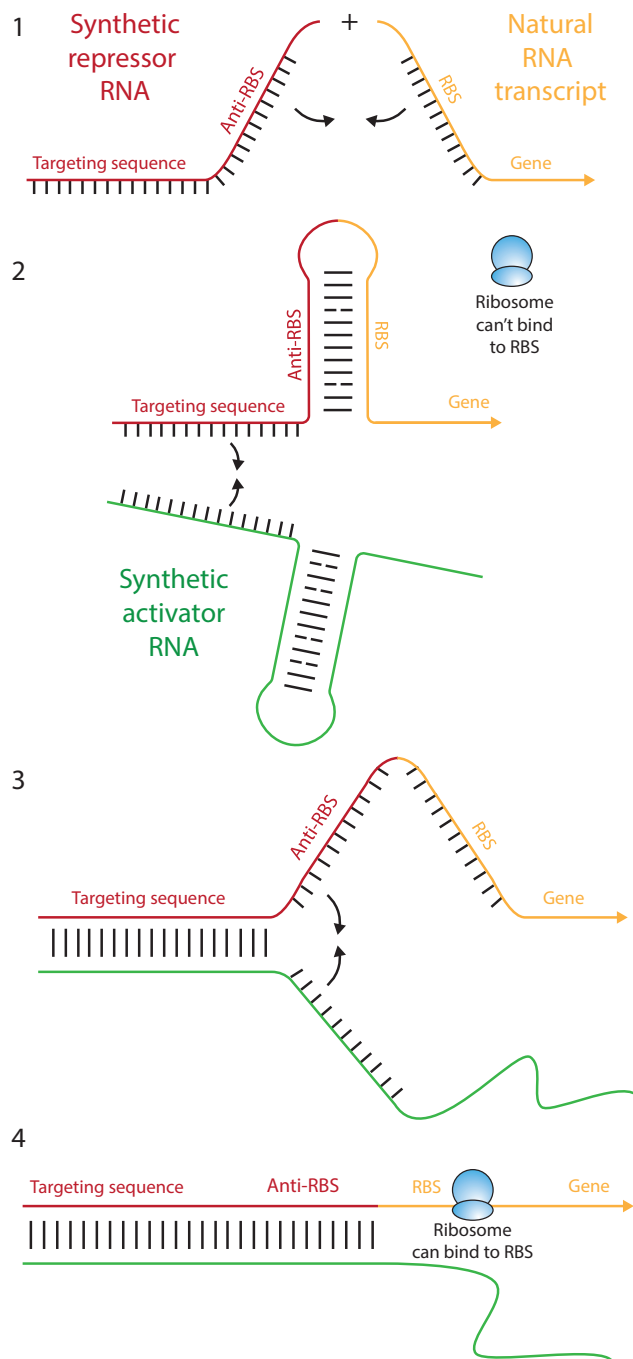
## WE'RE TRYING TO AFFECT ONE PATHWAY IN THE MIDDLE OF A SEA OF PATHWAYS.

“The idea,” Unkefer says, “is to bypass billions of years of evolution and engineer entire bacterial metabolic pathways to a particular end.” That end could be either the production or consumption of particular products that we humans either want more of (e.g. fuels) or less of (e.g. toxins).

Consider a generic metabolic pathway that is mediated by a series of enzymes: Enzyme-A performs some sort of action on chemical-A, typically adding something or taking something away, yielding a product that is a new chemical, which gets modified next by enzyme-B. Enzyme-B converts the chemical into something that gets modified next by enzyme-C and so on in a cascade of sequential reactions. Unkefer and his team want to engineer that whole thing. With that goal in mind, the team has pioneered the development of a new class of artificial regulatory RNA molecules, which it calls riboregulators, and which are entirely separate from innate riboswitches.

Whereas riboswitches modulate gene expression by allowing or disallowing the production of a gene transcript, riboregulators operate by allowing or disallowing translation of gene transcripts into protein by ribosomes. In order for a ribosome to translate a gene transcript into a protein, the ribosome needs to attach itself to the transcript. But it can't just clamp on any old place; it has to find and clamp on at a very particular sequence, conveniently called a ribosome-binding site (RBS), which usually reads AGGAGG and is located just before the gene on the transcript. So it stands to reason that if ribosomes can't find the RBS, they can't clamp on, and the protein can't get made. That's the strategy the Los Alamos riboregulator team tried, and it worked.

First, the scientists synthesized a short sequence of RNA that contains, among other things, a segment mostly complementary to the RBS, having maybe five perfect matches and one



How a riboregulator uses the ribosome binding sequence (RBS) of a gene transcript (yellow) to regulate ribosome access and consequent protein production: (1) The Los Alamos team attaches a synthetic piece of RNA (red) containing a targeting sequence and an anti-RBS sequence to the RBS at the end of the gene transcript. The anti-RBS sequence binds to the RBS, making ribosomes unable to attach and preventing protein production. (2) Next, they add a synthetic activator RNA (green) to bind to the targeting sequence of the repressor RNA and maneuver a neighboring segment, which is a better match to the anti-RBS sequence, into proximity to the anti-RBS. (3) The anti-RBS breaks bonds with the RBS and forms new bonds with the better-matched segment (green pairs with red), making the RBS (4) available for ribosome attachment to produce a protein. The amount of activator added allows researchers to fine-tune how much protein gets made.

flexible match to the AGGAGG of the RBS. Next, they attached the synthetic RNA like a tail to an actual gene transcript right next to the RBS. This put the anti-RBS sequence and the RBS near one another on the same strand of RNA, allowing them



A trio of Los Alamos riboregulators:  
(left to right) Cliff Unkefer, Karissa  
Sanbonmatsu, and Scott Hennelly.

to pair with each other, which prevents roaming ribosomes from clamping on. That's the repressor function: production of the protein encoded on that transcript has been repressed. But there's also an activator function that can be dialed up or down, making the system tunable, which is why it's a *riboregulator* and not a *riboswitch*.

The activator function comes from a second synthetic RNA that competes for the anti-RBS of the repressor, repressing the repressor and resulting in activation. The RNA tail that was attached to the transcript to repress it also contained a segment called a targeting sequence, which acts like a trap to attract and anchor the activator RNA. The activator is free-floating and not attached to anything, but it has a segment that is a perfect match to the targeting sequence of the repressor RNA, so the two strands eventually pair up. Now that the activator is bound to the repressor, a segment of

## LIKE WITH A SOUND MIXING BOARD, YOU CAN'T JUST TURN EVERYTHING ON FULL BLAST AND HAVE IT SOUND GOOD.

the activator that is a perfect match to the anti-RBS is brought into proximity to the anti-RBS. Because the anti-RBS was a pretty good match to the RBS but not a perfect match, when the perfect match sequence on the activator comes along, the anti-RBS breaks bonds with the RBS and pairs up with the better-matched sequence on the activator RNA. Now the RBS is available, and ribosomes can come at will to crank out protein molecules.

How much activator RNA is around determines how much protein gets made, and how much protein gets made affects all the downstream steps of the metabolic pathway. In the previous generic enzyme cascade, where enzyme-A turns chemical-A into chemical-B and so on, riboregulators could be used to tweak and adjust each step in that cascade. So far, the team has successfully managed to riboregulate two genes in the same cell. But as Hennelly points out, "If there are ten achievable levels of expression for each of five different proteins, that's 100,000 different unique combinations. That's the tunability we're talking about. That's what's new and what I'm excited about."

So the riboregulator team's vision of engineering entire metabolic pathways isn't too far out. Now they're working out the details of combination. "It's kind of like a sound mixing board," Hennelly says, "you can't just turn everything on full blast and expect it to sound good. There are a lot of controls. Sometimes when you turn one up you have to turn another one down."

### It's a ribo world

So far, the riboregulators have worked exactly as the team had hoped. But beyond the scientific satisfaction of one's theories and hard work panning out, riboswitches and riboregulators have tangible gains to offer as well. Both mechanisms are powerful tools for synthetic biology applications. One example is bioremediation: making bacteria metabolize chemicals that they ordinarily wouldn't consume in order to clean up environmental messes. Another example is bioenergy—engineering bacteria to produce fuels or help remove carbon dioxide from the atmosphere.

Riboswitches are also promising targets for new antibiotics. Because they are in bacterial cells but not in human cells, a riboswitch-targeting drug would be handy in fighting infection. And the high specificity of riboswitches for their particular ligands would make the drugs easily targeted to the bacterial gene of choice.

The regulation of gene expression is outrageously complex and frequently relies on the unique characteristics and abilities of RNA. So although DNA is the handsome figurehead of genes and genetics, RNA is the unsung hero. Scrappy and resourceful, RNA acts as field crew, moving team, courier service, fact checker, and gatekeeper. And in the hustling and bustling metropolis of a living cell, that truly is the stuff of life. **LDRD**

—Eleanor Hutterer

### More RNA research at Los Alamos

- **Ribosomes: the smartest living nanomachine**  
<http://www.lanl.gov/discover/publications/1663/issues-archive/august2008.pdf>
- **Counting RNA molecules to monitor bacteria**  
<http://www.lanl.gov/discover/news-stories-archive/2013/June/disease-causing-organisms.php>